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I. Biotinylation Reagents

The highly specific interaction of avidin with the small vitamin biotin can be a useful tool in designing assay, detection, and targeting systems for biological analytes (see

Chapter 13). The extraordinary affinity of avidin's interaction with biotin allows biotin-containing molecules in complex mixtures to be discretely bound with avidin conjugates. If the avidin–biotin complex contains detection components, then the targeted analytes can be located or quantified. This assay concept is made possible through the ability of biotin to be covalently attached to other targeting molecules, such as antibodies. In this sense, biotin derivatives may be prepared that contain reactive portions able to couple with particular functional groups in proteins and other molecules. Biotin modification of secondary molecules, called "*biotinylation,*" results in covalent derivatives containing one or more bicyclic biotin rings extending from the parent structure. These biotinylation sites are still capable of binding avidin or streptavidin with the specificity and avidity of free biotin in solution. Since the biotin components are relatively small, macromolecules can be modified with these reagents without significantly affecting their physical or chemical properties (Della-Penna *et al.*, 1986).

The basic design of a biotin-labeling compound is illustrated in Fig. 245. Common to all such modification reagents is the presence of the bicyclic biotin ring at one end of the structure and a reactive functional group at the other end that can be used to couple with other molecules. Biotinylation reagents also possess various cross-bridges or spacer groups built off the valeric acid side chain of the molecule. As the binding sites for biotin on avidin and streptavidin are pockets buried about 9 Å beneath the surface of the proteins, spacers can affect the accessibility of biotinylated compounds for efficiently binding avidin or streptavidin conjugates (Green *et al.*, 1971). In some applications, the use of a long spacer arm in the biotinylation reagent will result in the greatest potential assay sensitivity. The rate of binding of an avidin or streptavidin probe to a biotinylated molecule also is affected by the length of spacer in the biotinylation reagent used. When longer spacers are utilized to make biotinylated macromolecules, it potentially can result in a five-fold greater rate of streptavidin interaction (Bonnard *et al.*, 1984).

Another variable to consider in choosing biotinylation reagents is the use of a biotin analog such as iminobiotin that has a moderated affinity constant in its binding of avidin or streptavidin (Section 3.1). Analogs may be useful if release of the avidin–

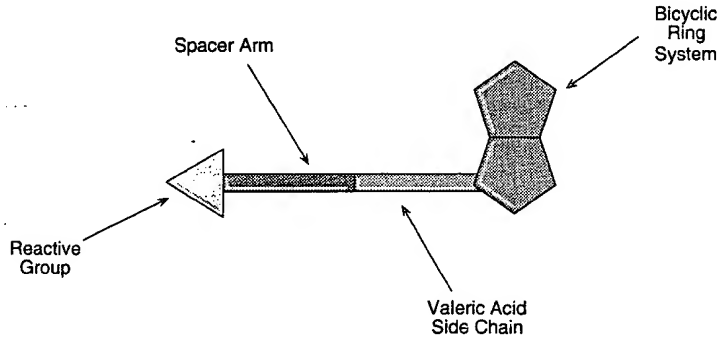


Figure 245 The basic design of a biotinylation reagent includes the bicyclic rings and valeric acid side chain of D-biotin at one end and a reactive functional group to couple with target groups at the other. Spacer groups may be included in the design to extend the biotin group away from modified molecules, thus ensuring better interaction capability with avidin or streptavidin probes.

biotin bond is important for isolating a targeted analyte. Using native biotin, the interaction with avidin is so strong that up to 6–8 *M* guanidine at pH 1.5 is required to break the bond, possibly causing extensive denaturation of any other complexed molecules. By contrast, iminobiotinylated molecules can be released simply by adjusting the pH down to 4.

The following sections discuss some of the more common biotinylation reagents available for modification of proteins and other biomolecules. Each biotin derivative contains a reactive portion (or can be made to contain a reactive group) that is specific for coupling to a particular functional group on another molecule. Careful choice of the correct biotinylation reagent can result in directed modification away from active centers or binding sites, and preserve the activity of the modified molecule.

3.1. Amine-Reactive Biotinylation Agents

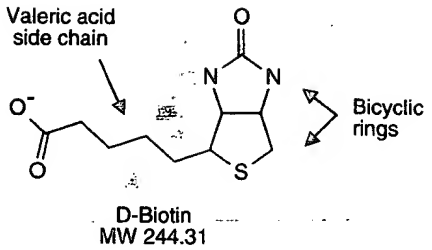
Amine-reactive biotinylation reagents contain functional groups off biotin's valeric acid side chain that are able to form covalent bonds with primary amines in proteins and other molecules. Two basic types are commonly available: NHS esters and carboxylates. NHS esters spontaneously react with amines to form amide linkages (Chapter 2, Section 1.4). Carboxylate-containing biotin compounds can be coupled to amines via a carbodiimide-mediated reaction using EDC (Chapter 3, Section 1.1).

D-*Biotin and Biocytin*

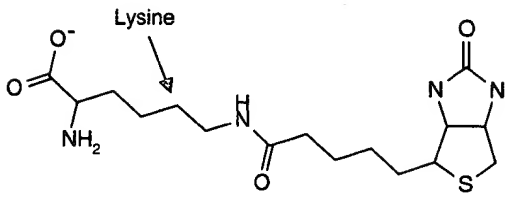
D-Biotin (hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid) is a naturally occurring growth factor present in small amounts within every cell. It is a key component in numerous processes involving carboxylation reactions, wherein it functions as a cofactor and transporter of CO_2 (coenzyme R). Biotin is mainly found covalently attached to lysine ε-amine groups of proteins via its valeric acid side chain. The compound was originally discovered through symptoms of deficiency caused by eating too many raw egg whites. Biotin (or vitamin H) was found to be complexed and inactivated by the egg-white protein avidin (Boas, 1927; du Vigneaud, 1940). Treatment with additional vitamin H alleviated the symptoms.

Biotin's interaction with the proteins avidin and streptavidin is among the strongest noncovalent affinities known ($K_a = 10^{15}\ M^{-1}$). The binding occurs between the bicyclic ring of biotin and a pocket within each of the four subunits of the proteins. The valeric acid portion is not involved or required for the interaction (Green, 1975; Wilchek and Bayer, 1988). This characteristic allows modification of the valeric acid side chain without affecting the binding potential toward avidin or streptavidin.

D-Biotin is thus the basic building block for constructing biotinylation reagents. The molecule may be attached directly to a protein via its valeric acid side chain or derivatized at this carboxylate with other organic components to create spacer arms and various reactive groups. Reaction of biotin with primary amine groups on proteins can be done using the water-soluble carbodiimide EDC (Chapter 3, Section 1.1). EDC activates the carboxylate to create a highly reactive, intermediate ester. The ester then can couple to amines to form stable amide bond derivatives (Fig. 246). Biotinylated molecules thus formed retain the ability to bind avidin or streptavidin with high affinity.



D-Biotin
MW 244.31



Biocytin
MW 372.48

The only potential deficiency in using D-biotin to modify directly a protein is the relatively short spacer arm afforded by the indigenous valeric acid group. Some applications may require longer spacers to maintain good binding potential toward avidin or streptavidin.

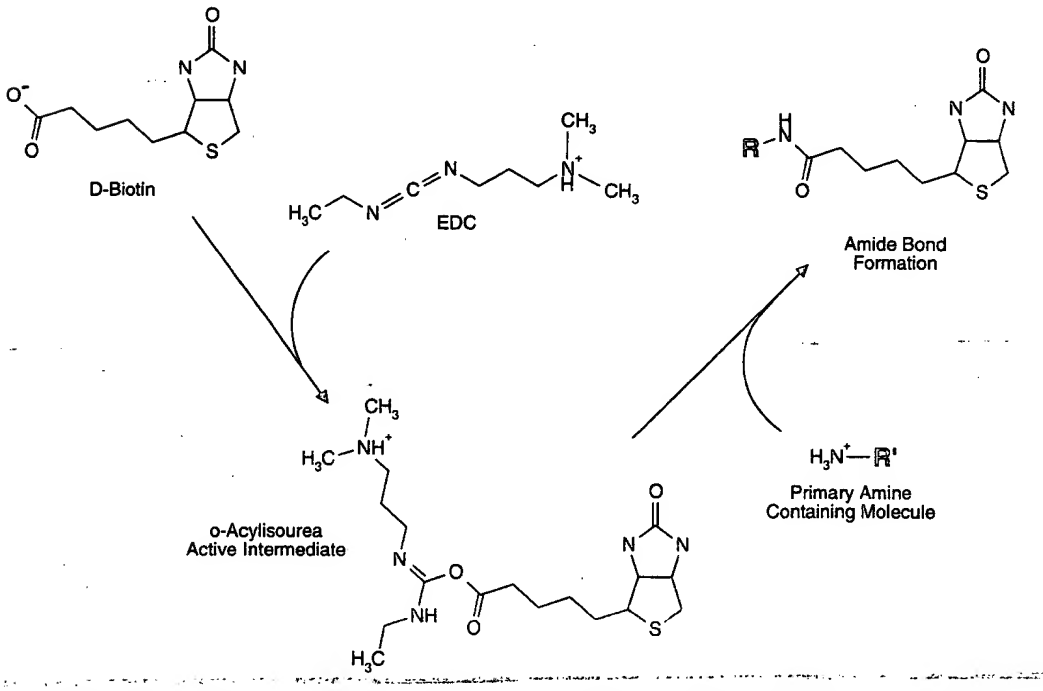


Figure 246 D-Biotin can be directly coupled to amine-containing molecules using the water-soluble carbodiimide EDC to form an amide bond linkage.

Biocytin is ε-*N*-biotinyl-L-lysine, a derivative of D-biotin containing a lysine group coupled at its ε-amino side chain to the valeric acid carboxylate. It is a naturally occurring complex of biotin that is typically found in serum and urine, and probably represents breakdown products of recycling biotinylated proteins. The enzyme biotinidase specifically cleaves the lysine residue and releases the biotin component from biocytin (Ebrahim and Dakshinamurti, 1986, 1987).

Biocytin has been used extensively as a labeling reagent for intracellular components within neurons (Horikawa and Armstrong, 1988; King *et al.*, 1989; Izzo, 1991; Granata and Kitai, 1992). It is particularly good for anterograde tracing studies in the central nervous system, since it can be easily injected into neurons using micropipettes. Subsequent visualization of biocytin locations may be done using an avidin–enzyme conjugate (Chapter 13).

Biocytin should not be used in a carbodiimide reaction to modify proteins or other molecules, since it contains both a carboxylate and an amine group. A carbodiimide-mediated reaction, as suggested for D-biotin, would cause self-conjugation and polymerization of this reagent.

Biocytin, however, can form the basis for constructing trifunctional cross-linking reagents (Chapter 6). The lysine component of the molecule contains a free carboxylate and an α-amine group that can be used to build spacers and reactive groups for cross-linking purposes. The biotin component is the third arm of the trifunctional system, retaining its ability to bind avidin probes after conjugation has occurred at its other two ends. Such a trifunctional derivative has been used to study the hormone binding site of the insulin receptor (Wedekind *et al.*, 1989). This compound, 4-azido-2-nitrophenyl-biocytin-4-nitrophenyl ester, contains an amine-reactive group and a photoreactive phenyl azide functional group (Chapter 6, Section 1). The nitrophenyl ester reacts with amines on proteins and other molecules to from stable amide linkages. Once a molecule is modified in this manner, it contains both a photosensitive group and a biotin handle for conjugation and detection, respectively. Interaction of the modified protein with another biospecific receptor and subsequent photolyzing with UV light results in covalently cross-linking. Localization and detection of the cross-linked molecules then can be done using an avidin or streptavidin conjugate. Another trifunctional compound, sulfo-SBED, also is based on a biocytin core (Chapter 6, Section 2).

NHS-Biotin and Sulfo-NHS-Biotin

The valeric acid carboxylate of D-biotin may be activated to an NHS ester for direct modification of amine groups in proteins and other molecules. NHS esters react by nucleophilic attack of an amine on the carbonyl group, releasing the NHS group, and forming a stable amide linkage (Chapter 2, Section 1.4) (Fig. 247). NHS-biotin is the simplest biotinylation reagent available. Modification reactions are carried out under mildly alkaline conditions and usually result in a high efficiency of biotin incorporation.

NHS-biotin is insoluble in aqueous environments. It must be first dissolved in organic solvent as a concentrated stock solution, and an aliquot added to an aqueous reaction medium to facilitate dissolution. Organic solvents such as DMF or DMSO are suitable for this purpose. Addition of an NHS-biotin solution to a reaction should not exceed a level of about 10% organic solvent in the buffer to avoid precipitation problems. Once added to the reaction medium, the NHS-biotin may appear as a

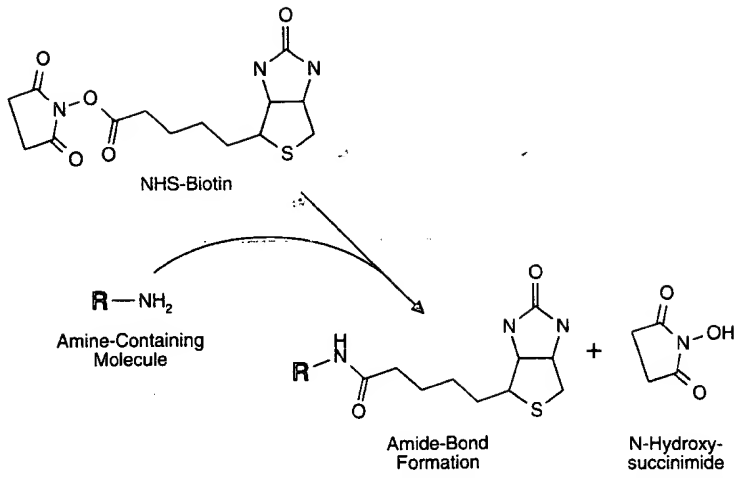
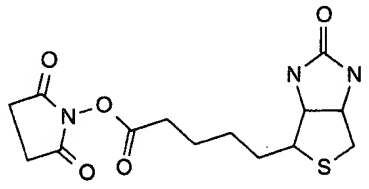
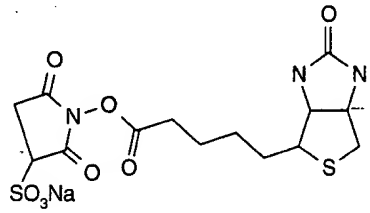


Figure 247 The active ester group of NHS-biotin reacts with amine-containing compounds to form amide bond linkages.

cloudy or hazy suspension, indicating incomplete solubility. However, such microdispersions still are effective at modification, often driving the bulk of the reagent into solution ast the NHS ester reacts. Biotinylation of peptides or other molecules that are water-insoluble may be done completely in organic solvent. For example, insulin can be biotinylated with NHS-biotin in an organic medium (Hofmann *et al.*, 1977).



NHS-Biotin
MW 341.38
13.5 Å



Sulfo-NHS-Biotin
MW 443.42
13.5 Å

A water-soluble analog of NHS-biotin containing a negatively charged sulfonate group on its NHS ring structure also is available. Sulfo-NHS-biotin may be added directly to aqueous reactions without the need for organic solvent dissolution. A concentrated stock solution may be prepared in water to facilitate the addition of a small quantity to a reaction, but hydrolysis of the NHS ester will occur at a rapid rate, so the solution must be used immediately.

The only disadvantage to the use of NHS-biotin or sulfo-NHS-biotin is the lack of a long spacer group off the valeric acid side chain. Because the binding site for biotin on avidin and streptavidin is somewhat below the surface of the proteins, some biotinylated molecules may not interact as efficiently with avidin (or streptavidin) as when longer cross-bridges are used (Green *et al.*, 1971; Bonnard *et al.*, 1984).

NHS esters of D-biotin have been used in many applications, including the biotinylation of rat IgE to study receptors on murine lymphocytes (Lee and Conrad, 1984), in the development of an immunochemical assay for a postsynaptic protein and its receptor (LaRochelle and Froehner, 1986a), in the study of plasma membrane domains by biotinylation of cell surface proteins in *Dictyostelium disoideum* amoebas (Ingalls *et al.*, 1986), and for the detection of blotted proteins on nitrocellulose membranes after transfer from polyacrylamide electrophoresis gels (LaRochelle and Froehner, 1986b).

The following protocol is a generalized method for the biotinylation of a protein using sulfo-NHS-biotin.

Protocol

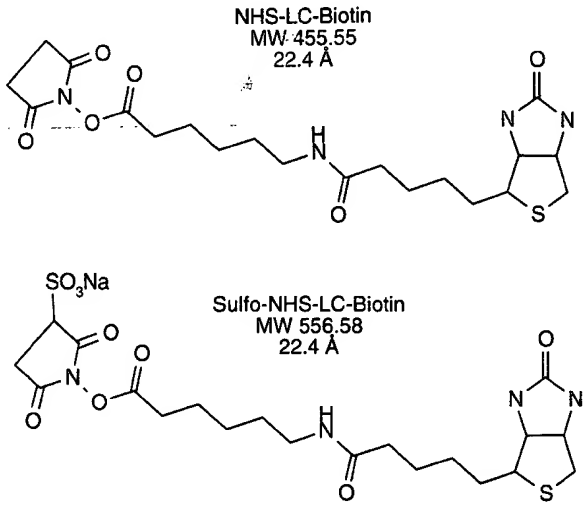
1. Dissolve the protein to be biotinylated in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 1–10 mg/ml.
2. Immediately before use, dissolve sulfo-NHS-biotin (Pierce) in water at a concentration of 20 mg/ml. Adjust the quantity of this stock solution to be prepared according to the amount of reagent needed to biotinylate the required amount of protein. The sulfo-NHS-biotin solution must be used immediately, since the NHS ester is subject to hydrolysis in aqueous environments.
3. With mixing, add a quantity of the sulfo-NHS-biotin solution to the protein solution to obtain a 12- to 20-fold molar excess of biotinylation reagent over the quantity of protein present. For instance, for an immunoglobulin (MW 150,000) at a concentration of 10 mg/ml, 20 µl of the sulfo-NHS-biotin solution (8×10^{-4} mmol) should be added per milliliter of antibody solution to obtain a 12-fold molar excess. For more dilute protein solutions (i.e., 1–2 mg/ml), increased amounts of biotinylation reagent may be required (i.e., 20-fold molar excess) to obtain similar incorporation yields as when using more concentrated protein solutions.
4. React for 30–60 min at room temperature.
5. Purify the biotinylated protein from excess reagent and reaction by-products by gel filtration using a column of Sephadex G-25 or by dialysis against PBS.

Determination of the degree of biotinylation can be done using the HABA assay (Chapter 13, Section 7).

NHS-LC-Biotin and Sulfo-NHS-LC-Biotin

NHS-LC-biotin is a derivative of D-biotin containing a spacer arm off the valeric acid side chain, terminating in an NHS ester. The compound is also known as succinimidyl-6-(biotinamido)hexanoate or NHS-X-biotin. The 6-aminocaproic acid spacer provides greater length between a convalently modified molecule and the bicyclic biotin rings. The total distance from an attached molecule to the biotin component is about 22.4 Å, significantly greater than the 13.5 Å length of NHS-biotin without a

spacer arm. This increased distance can result in better binding potential for avidin or streptavidin probes, because the binding sites on these proteins are buried relatively deep inside the surface plane.



The NHS ester end of NHS-LC-biotin reacts with amine groups in proteins and other molecules to form stable amide-bond derivatives (Fig. 248). Optimal reaction conditions are at a pH of 7–9. Avoid amine-containing buffers, which may compete in the acylation reaction. NHS-LC-biotin is insoluble in aqueous reaction conditions and must be solubilized in organic solvent prior to the addition of a small quantity to a

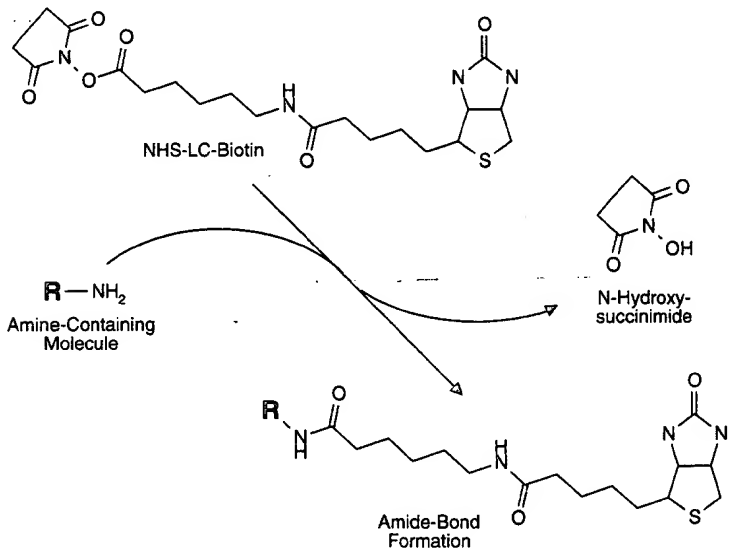


Figure 248 NHS-LC-biotin provides an extended spacer arm to allow greater distance between the biotin rings and a modified molecule. Reaction with amines forms amide linkages.

buffered reaction. Preparation of concentrated stock solutions may be done in DMF or DMSO. Nonaqueous reactions also may be done with this reagent for the modification of molecules insoluble in water. The molar ratio of NHS-LC-biotin to a protein in a reaction can be from about 2:1 to about 50:1, with higher levels resulting in better incorporation yields (Gretch *et al.*, 1987).

In a study comparing NHS-LC-biotin with two other derivatives of biotin, NHS-SS-biotin (Section 3.1) and biotin hydrazide (Section 3.3), it was found that modification through amines on monoclonal antibodies resulted in 2.5 times more activity in binding a streptavidin–agarose affinity column than when modification of carbohydrate residues using hydrazide chemistry was done (Gretch *et al.*, 1987). This was probably due to the greater abundance of amino groups over polysaccharide residues on these antibodies.

NHS-LC-biotin can be used to add a biotin tag to monoclonal antibodies directed at certain tumor antigens. The biotinylated monoclonals are allowed to bind to the tumor cell surfaces *in vivo*, and subsequent administration of an avidin or a streptavidin conjugate can form the basis for inducing cytotoxic effects or creating traceable complexes for use in imaging techniques (Hnatowich *et al.*, 1987).

The reagent also has been used in a unique tRNA-mediated method of labeling proteins with biotin for nonradioactive detection of cell-free translation products (Kurzchalia *et al.*, 1988), in creating one- and two-step noncompetitive avidin–biotin immunoassays (Vilja, 1991), for immobilizing streptavidin onto solid surfaces using biotinylated carriers with subsequent use in a protein avidin-biotin capture system (Suter and Butler, 1986), and for the detection of DNA on nitrocellulose blots (Leary *et al.*, 1983).

Sulfo-NHS-LC-biotin, a water-soluble analog of NHS-LC-biotin, also is available (Pierce), and contains a negatively charged sulfonate group on its NHS ring structure. The presence of the negative charge creates enough polarity within the molecule to allow direct solubility in aqueous reaction mediums. All other properties of the sulfonated version of the reagent are the same as those of NHS-LC-biotin.

The following protocol is a suggested method for the biotinylation of proteins with either NHS-LC-biotin or sulfo-NHS-LC-biotin.

Protocol

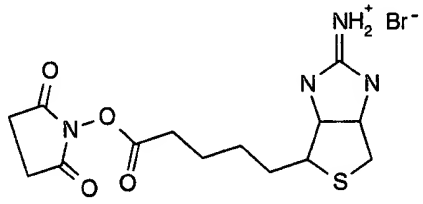
1. Dissolve the protein to be biotinylated in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Dissolve NHS-LC-biotin (Pierce) in DMF at a concentration of 40 mg/ml. This stock solution is stable for reasonable periods, although long-term storage is not recommended. For use of the water-soluble sulfo-NHS-LC-biotin, a stock solution may be prepared in water, or the solid reagent may be directly added to the reaction mixture. If a solution in water is made to facilitate the addition of a small quantity of reagent to a reaction, then the solution should be prepared quickly and used immediately to prevent hydrolysis of the NHS ester. Sulfo-NHS-LC-biotin may be dissolved in water at a concentration of 20 mg/ml.
3. Add 50 µl of the NHS-LC-biotin solution in DMF to each milliliter of the protein solution in two aliquots apportioned 10 min apart. Alternatively, add a quantity of the sulfo-NHS-biotin solution prepared in water to the protein solution to obtain a 12- to 20-fold molar excess of biotinylation reagent over the quantity of

protein present. For instance, for an immunoglobulin (MW 150,000) at a concentration of 10 mg/ml, 20 µl of the sulfo-NHS-biotin solution (8×10^{-4} mmol) should be added per milliliter of antibody solution to obtain a 12-fold molar excess.

4. React for a total of 30–60 min at room temperature or several hours at 4°C.
5. Remove unreacted biotinylation reagent and reaction by-products by gel filtration using a column of Sephadex G-25 or dialysis against PBS.
6. Assay the level of biotin incorporation using the HABA dye procedure (Chapter 13, Section 7).

NHS-Iminobiotin

NHS-iminobiotin is *N*-hydroxysuccinimido-2-iminobiotin, the guanidino analog of NHS-biotin that has a lower affinity constant for binding avidin or streptavidin. Iminobiotin replaces the 2-oxo-imidazole upper ring structure of D-biotin with a 2-imino-imidazole structure, causing moderated interaction with the avidin or streptavidin binding sites. This biotin analog can be used in situations requiring mild dissociation of the avidin–biotin complex. Normally, breaking the avidin–biotin interaction requires 6–8 *M* guanidine hydrochloride at a pH of 1.5, an environment too severe for most proteins to maintain native structure or recover activity. Iminobiotin, by contrast, can be bound to avidin or streptavidin at a pH wherein the guanidino group is unprotonated and thus uncharged. Binding occurs at pH values above 9.5 (typically done at pH 11), and elution can be accomplished simply by changing the pH to 4—an environment that protonates the 2-imino group and creates a positive charge—effectively dissociating the interaction (Fig. 249).



NHS-Iminobiotin
N-Hydroxysuccinimido-
iminobiotin hydrobromide
MW 421.32
13.5 Å

NHS-iminobiotin can be used to label amine-containing molecules with an iminobiotin tag, providing reversible binding potential with avidin or streptavidin. The NHS ester reacts with proteins and other amine-containing molecules to create stable amide-bond derivatives (Fig. 250). An iminobiotinylated molecule then can be used to target and purify other components in biological samples. For instance, a targeting molecule, such as an antibody, can be iminobiotinylated and allowed to bind its target in complex mixtures (such as tissue sections, cell extracts, or homogenates). The antibody–antigen complex subsequently can be purified using an affinity column of immobilized avidin with binding at pH 10–11 and simple elution at pH 4 (Orr, 1981; Zeheb *et al.*, 1983). The relatively mild elution conditions allows recovery of the bound antigen without exposure to severe denaturing conditions.

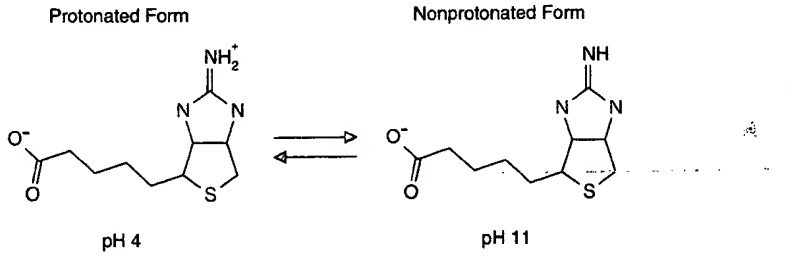


Figure 249 At pH 4, the protonated form of iminobiotin does not interact with the binding sites on avidin or streptavidin. At pH 11, the imino group is unprotonated and regains binding capability toward these proteins.

The iminobiotin–avidin interaction also can be utilized in the opposite approach. Immobilized iminobiotin affinity columns can be used to purify avidin- or streptavidin-containing complexes under mild elution conditions (Hofmann *et al.*, 1980).

NHS-iminobiotin is insoluble in aqueous solution. It can be dissolved in organic solvent (DMF) prior to addition of a small aliquot to a buffered reaction medium. Do not exceed 10% DMF in the reaction, to avoid protein precipitation problems. Optimal conditions for protein derivatization include non-amine-containing buffers at a pH of 7–9. The following protocol is a suggested method for labeling antibodies with NHS-iminobiotin. Some optimization may have to be done for particular derivatization needs.

Protocol

1. Dissolve the antibody to be modified in 50 m*M* sodium borate, pH 8, at a concentration of 5 mg/ml.

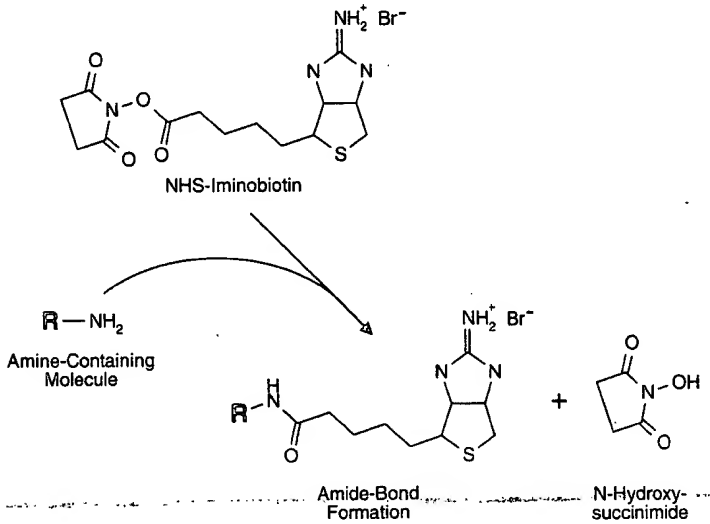
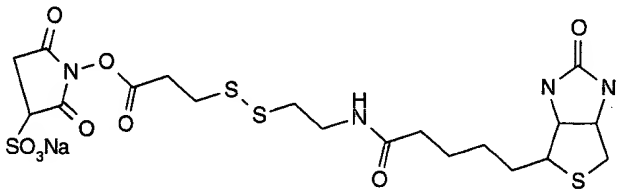


Figure 250 NHS-iminobiotin can be used to label amine-containing molecules, creating amide linkages.

2. Dissolve NHS-iminobiotin in DMF at a concentration of 1 mg/ml. Prepare fresh.
3. Add 100 µl of the NHS-iminobiotin solution to each milliliter of the antibody solution. Mix well to dissolve. Note: some turbidity may be present in the reaction due to incomplete dissolution of the NHS-iminobiotin. The solution may look cloudy or have a microparticulate suspension present. This is normal for many water-insoluble reagents when added to an aqueous solution in an organic solvent. As the reaction takes place, the NHS-iminobiotin will be driven into solution, both by coupling to the protein and by hydrolysis of the NHS ester.
4. React for 30–60 min at room temperature or for 3 h at 4°C.
5. Remove unreacted NHS-iminobiotin and reaction by-products by dialysis or gel filtration using a column of Sephadex G-25.

Sulfo-NHS-SS-Biotin

Sulfo-NHS-SS-biotin (also known as NHS-SS-biotin) is sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate, a long-chain cleavable biotinylation reagent that can be used to modify amine-containing proteins and other molecules (Pierce). The cross-bridge of the compound provides a 24.3-Å spacer arm that creates plenty of distance between the modified molecule and the biotin end. Using a long-chain biotinylation reagent can increase the efficiency of biotinylated molecules to bind avidin or streptavidin conjugates, thus enhancing the potential sensitivity of assay systems.



Sulfo-NHS-SS-Biotin
Sulfosuccinimidyl-2-(biotinamido)-
ethyl-1,3-dithiopropionate
MW 606.7
24.3 Å

After sulfo-NHS-SS-biotin-modified molecules are allowed to interact with avidin or streptavidin probes, the formed complexes can be cleaved at the disulfide bridge by treatment with 50 m*M* DTT. Reduction releases the biotinylated component from the avidin or streptavidin detection reagent. The use of disulfide biotinylation reagents thus provides much gentler conditions to break the complex than would be required if the avidin-biotin interaction itself were disrupted (which dissociates only at 6–8 *M* guanidine, pH 1.5).

The use of a cleavable biotinylation reagent also provides a means to purify targeted molecules using affinity chromatography on a column of immobilized avidin or streptavidin. For instance, an antibody modified with sulfo-NHS-SS-biotin can be allowed bind its target in complex mixtures (such as tissue sections, cell extracts, or homogenates). The antibody–antigen complex subsequently can be isolated using an affinity column of immobilized avidin or streptavidin. Elution from the column with DTT

breaks the disulfide bonds, releasing the antibody and its bound antigen. The isolation of Herpes virus proteins (Gretch *et al.*, 1987) and the recovery of DNA binding proteins (Shimkus *et al.*, 1985) were both done using this approach.

Due to the presence of the negatively charged sulfonate group, sulfo-NHS-SS-biotin is a water-soluble biotinylation reagent that may be added directly to aqueous reactions without prior dissolution in organic solvent. For the addition of small quantities of reagent, the compound may be dissolved in water, and an aliquot transferred to the reaction medium. If an aqueous stock solution of sulfo-NHS-SS-biotin is prepared, it must be dissolved rapidly and used immediately to prevent hydrolysis of the active ester. The NHS ester reaction forms stable amide-bond linkages with amine-containing proteins and other molecules (Fig. 251). Optimal conditions for the NHS ester reaction include a pH of 7–9, avoidance of any amine-containing buffers or other components that may compete in the reaction (including imidazole buffers, which catalyze hydrolysis of these esters), and avoidance of reducing agents that could cleave the disulfide bridge.

The following protocol is a suggested method for biotinylating antibody molecules with sulfo-NHS-SS-biotin. Some optimization may have to be done with each application to ensure good biotin incorporation with retention of antigen binding activity. Other proteins and amine-containing molecules may be biotinylated using similar conditions.

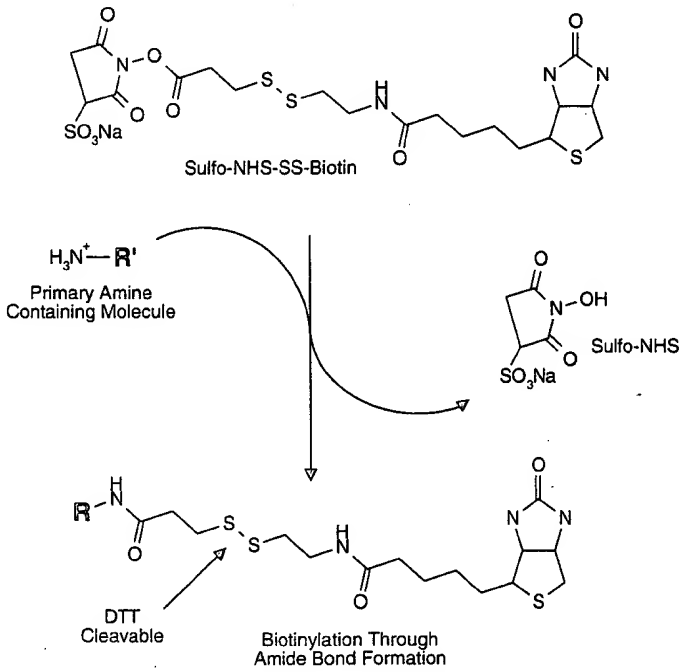


Figure 251 Sulfo-NHS-SS-biotin reacts with amine groups to form amide bonds. The biotin group can be later cleaved off the modified molecule by reduction of its internal disulfide linkage.

Protocol

1. Dissolve the antibody to be biotinylated in 50 m*M* sodium bicarbonate, pH 8.5, at a concentration of 10 mg/ml. Other buffers and pH conditions between pH 7 and 9 can be used as long as no amine-containing buffers like Tris are present. Avoid also the presence of disulfide-reducing agents that can cleave the disulfide group of the biotinylation reagent.
2. Add 0.3 mg of sulfo-NHS-SS-biotin (Pierce) to each milliliter of the antibody solution. To measure small amounts of the biotinylation reagent, it may be first dissolved in water at a concentration of at least 1 mg/ml. Immediately transfer the appropriate amount to the antibody solution. This level of sulfo-NHS-SS-biotin addition represents about an eight-fold molar excess over the amount of antibody present. This should result in a molar incorporation of approximately two to four biotins per immunoglobulin molecule.
3. React for 30–60 min at room temperature or for 2–4 h at 4°C.
4. Remove unreacted biotinylation reagent and reaction by-products by dialysis or gel filtration using a column of Sephadex G-25.

3.2. Sulfhydryl-Reactive Biotinylation Agents

Sulfhydryl-reactive biotinylation reagents allow modification at cysteine —SH groups or at sites of specific thiolation within proteins and other molecules. Targeting sulfhydryls for modification, as opposed to amines, usually results in more limited derivatization, often away from active centers or binding sites. Directed coupling of biotin in this manner can aid in preserving activity. For instance, antibodies may be cleaved by reduction at their disulfide groups in the hinge region, forming free sulfhydryls removed from the antigen-binding site (Chapter 10, Section 1.1). Biotinylation at these sites produces a derivative that can bind efficiently both antigen and avidin or streptavidin probes without steric hindrance.

Sulfhydryl groups also can be added to 5′-phosphate end of DNA probes (Chapter 17, Section 2.2). Biotinylation at these sites avoids disruption of base-pairing with complementary DNA targets, since the point of modification is restricted to a single end position on the oligonucleotide.

The following sections discuss three sulfhydryl-reactive biotinylation reagents that utilize maleimide, pyridyl disulfide, and iodoacetyl reactive groups, respectively. The maleimide and iodoacetyl options produce nonreversible, covalent thioether linkages with target —SH groups. The pyridyl disulfide chemistry results in disulfide bonds that are reversible through cleavage with a reducing agent.

Biotin-BMCC

Biotin-BMCC is 1-biotinamido-4-[4′-(maleimidomethyl)cyclohexane-carboxamido]butane, a biotinylation reagent containing a maleimide group at the end of an extended spacer arm (Pierce). The maleimide end reacts with sulfhydryl groups in proteins and other molecules to form stable thioether linkages (Fig. 252). The reaction is highly specific for —SH groups in the range of pH 6.5 to 7.5. The long spacer arm (32.6 Å) provides more than enough distance between modified molecules and the bicyclic biotin end to allow efficient binding of avidin or streptavidin probes.

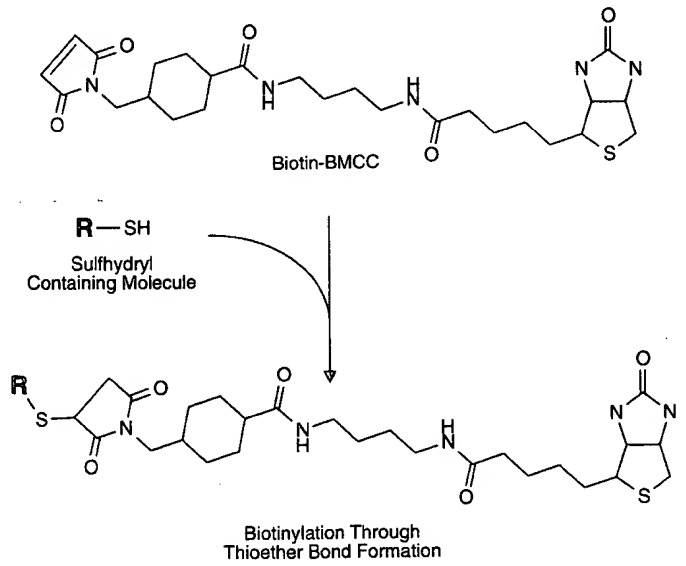
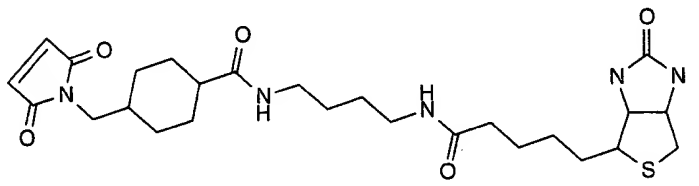


Figure 252 Biotin-BMCC provides sulfhydryl reactivity through its terminal maleimide group. The reaction creates a stable thioether linkage.



Biotin-BMCC
1-Biotinamido-4-[4'-(maleimidomethyl)-
cyclohexane-carboxamido]butane
MW 533.69
32.6 Å

The reagent is similar to another maleimide-containing biotinylation reagent, 3-(*N*-maleimidopropionyl) biocytin, a compound used to detect sulfhydryl-containing molecules on nitrocellulose blots after SDS–electrophoresis separation (Bayer *et al.*, 1987b). Biotin-BMCC should be useful in similar detection procedures.

Biotin-BMCC is insoluble in water and must be dissolved in an organic solvent prior to addition to an aqueous reaction mixture. Preparing a concentrated stock solution in DMF or DMSO allows transfer of a small aliquot to a buffer reaction. The upper limit of biotin-BMCC solubility in DMSO is approximately 33 m*M* or 17 mg/ml. In DMF, it is only soluble to a level of about 7 m*M* (4 mg/ml). On addition of an organic solution of the reagent to an aqueous environment (do not exceed 10% organic solvent in the aqueous medium to prevent protein precipitation), biotin-BMCC may form a microemulsion. This is normal and during the course of the reaction the remainder of the compound will be driven into solution as it couples or hydrolyzes.

The required sulfhydryl groups for biotin-BMCC modification may be indigenous

in molecules, formed through reduction of disulfides, or created by the use of thiolation reagents (Chapter 1, Section 4.1). At physiological pH values the rate of the maleimide reaction toward sulfhydryls is almost 1000-fold faster than its reaction toward amines. However, at higher pH values the maleimide will couple to amines quite readily (Ishi and Lehrer, 1986; Wu *et al.*, 1976). Maleimides also can undergo a ring-opening hydrolysis reaction that increases in rate with pH, effectively inactivating the functional group.

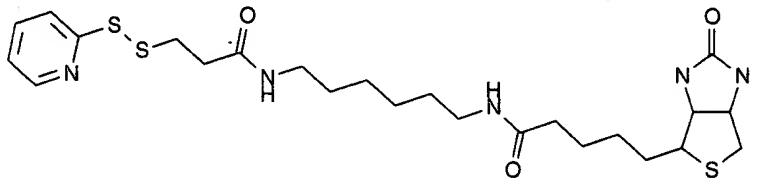
The following protocol is a suggested method for modifying sulfhydryl-containing proteins with biotin-BMCC. Some optimization of biotinylation levels may have to be done for particular applications.

Protocol

1. Dissolve the protein to be biotinylated (containing one or more free sulfhydryls) in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.2, at a concentration of 2.5 mg/ml.
2. Dissolve biotin-BMCC (Pierce) in DMSO at a concentration of 5 mg/ml.
3. Add 100 μl of the biotin-BMCC solution to each milliliter of the protein solution. Mix well.
4. React for at least 2 h at room temperature.
5. Remove excess biotinylation reagent and reaction by-products by dialysis or gel filtration using a column of Sephadex G-25 (Pharmacia).

Biotin-HPDP

Biotin-HPDP is *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Pierce). The reagent contains a 1,6-diaminohexane spacer group that is attached to biotin's valeric acid side chain. The terminal amino group of the spacer is further modified via an amide linkage with the acid of SPDP (Chapter 5, Section 1.1) to create a terminal, sulfhydryl-reactive functional group. The pyridyl disulfide end of biotin-HPDP can react with free —SH groups in proteins and other molecules to from a disulfide bond with loss of pyridine-2-thione (Fig. 253). This leaving group may be monitored by its characteristic absorbance at 343 nm to assess the level of biotinylation. However, since its extinction coefficient is rather low (about $8 \times 10^3\ M^{-1}cm^{-1}$), small-scale biotinylations may not be quantifiable using this technique.



Biotin–HPDP
N-[6-(Biotinamido)hexyl]-3'-
(2'-pyridyldithio)propionamide
MW 539.77
29.2 Å

Modifications done with biotin-HPDP produce biotinylated compounds with long spacer arms (29.2 Å), ensuring good binding efficiency with avidin or streptavidin

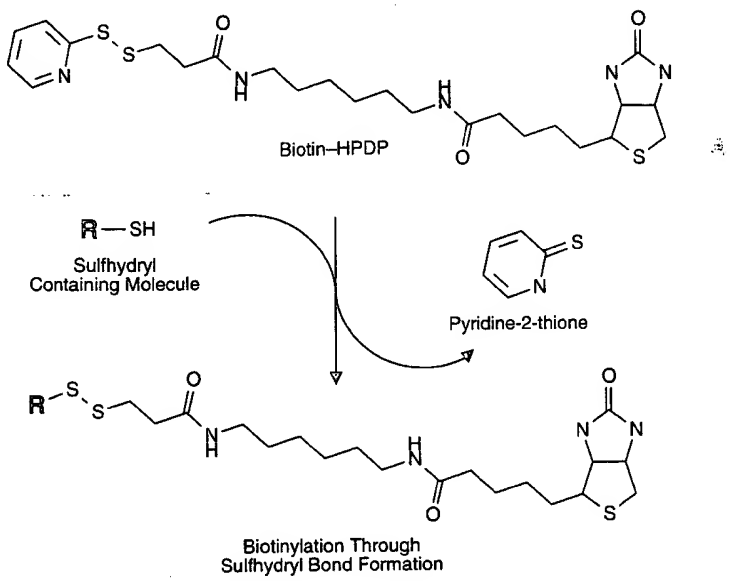


Figure 253 Biotin-HPDP reacts with sulfhydryl-containing molecules through its pyridyl disulfide group, forming reversible disulfide bonds. The biotin group may be released from modified molecules by reduction with DTT.

probes. After coupling to sulfhydryl-containing molecules, the biotin-HPDP component can be cleaved by treatment with disulfide-reducing agents, such as DTT. Breaking this bond releases the biotin modifications and regenerates the original sulfhydryl-containing molecule. This cleavability also provides a means of recovering target complexes after purification of the biotinylated molecules by affinity chromatography on immobilized avidin or streptavidin. Thus biotin-HPDP-modified antibodies directed against some specific cellular antigen can aid in the isolation of targeted components using affinity chromatography followed by elution with a disulfide reductant.

Using a similar approach, Clq has been modified with biotin-HPDP and allowed to interact with its specific receptor. Subsequent purification of the Clq receptor was accomplished through cleavage of the disulfide bridge of the biotinylation reagent (Ghebrehiwet *et al.*, 1988).

Biotin-HPDP is water-insoluble and therefore must be dissolved in an organic solvent prior to addition to an aqueous reaction medium. Suitable solvents include DMSO and DMF. Concentrated stock solutions may be prepared in DMSO, and a small aliquot transferred to a buffered reaction solution. Do not add more than 10% organic solvent to the aqueous reaction to prevent precipitation or denaturation of biological molecules. After addition, a microemulsion may result. This is normal for many water-insoluble reagents. The solution usually will become clear during the course of the reaction. Optimal conditions for the disulfide interchange reaction include a pH range of 6–9 in buffer systems that do not contain any extraneous sulfhydryl compounds. If reducing agents such as DTT or 2-mercaptoethanol are used to create sulfhydryls in the protein to be biotinylated, these must be completely removed by dialysis or gel filtration before reacting with biotin-HPDP.

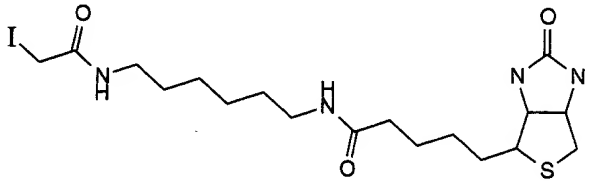
A suggested protocol for the use of biotin-HPDP in the modification of sulfhydryl-containing proteins follows. Similar procedures may be used when biotinylating other molecules.

Protocol

1. Dissolve the sulfhydryl-containing protein to be biotinylated in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.2, at a concentration of at least 2 mg/ml.
2. Dissolve biotin-HPDP (Pierce) in DMSO at a concentration of 4 m*M* (2.1 mg/ml).
3. Add 100 µl of the biotin-HPDP stock solution to each milliliter of the protein solution. Mix well.
4. React for 90 min at room temperature.
5. Purify the biotinylated protein by dialysis or gel filtration using a column of Sephadex G-25. The PBS/EDTA buffer described in step 1 is suitable for either operation.

Iodoacetyl-LC-Biotin

Iodoacetyl-LC-biotin is *N*-iodoacetyl-*N*-biotinylhexylenediamine, a sulfhydryl-reactive biotinylation agent (Pierce). The reagent contains a 1,6-diaminohexane spacer group that is attached to biotin's valeric acid side chain. The terminal amino group of the spacer is further modified via an amide linkage with an iodoacetyl group to provide the sulfhydryl reactivity. Coupling to sulfhydryl-containing proteins or other molecules creates nonreversible thioether bonds (Fig. 254). Modifications done with iodoacetyl-LC-biotin produce biotinylated compounds with sufficiently long spacer arms (27.1 Å) to ensure excellent binding potential with avidin or streptavidin probes.



Iodoacetyl-LC-Biotin
N-Iodoacetyl-N-biotinylhexylenediamine
MW 510.42
27.1 Å

Iodoacetyl-LC-biotin is water-insoluble and therefore must be dissolved in an organic solvent prior to addition to an aqueous reaction medium. Suitable solvents include DMSO and DMF. Concentrated stock solutions may be prepared in DMSO, and a small aliquot transferred to a buffered reaction solution. Do not add more than 10% organic solvent to the aqueous reaction to prevent precipitation or denaturation of biological molecules. After addition, a microemulsion may result. This is normal for many water-insoluble reagents. The solution usually will become clear during the course of the reaction. Optimal conditions for coupling using iodoacetyl-containing reagents include a pH range of 7.5–8.5 in buffer systems that do not contain any

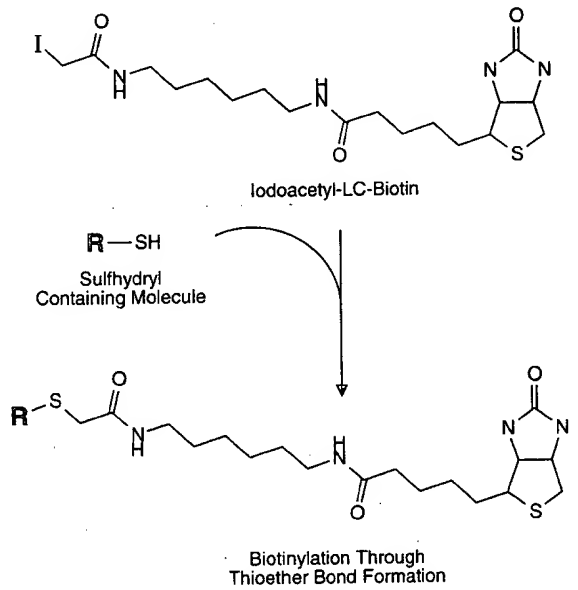


Figure 254 This biotinylation reagent reacts with sulfhydryl groups through its iodoacetamide end to form thioether bonds.

extraneous sulfhydryl compounds. In addition, protect all solutions containing iodoacetyl-LC-biotin from light, since photolysis may cause liberation of iodine, degrading the activity of the compound and possibly causing modification of tyrosine or histidine residues.

Iodoacetyl-LC-biotin has been used to localize the SH_1 thiol of myosin by use of an avidin–biotin complex visualized by electron microscopy (Sutoh *et al.*, 1984) and to determine the spatial relationship between SH_1 and the actin binding site on the myosin subfragment-1 surface (Yamamoto *et al.*, 1984).

The following protocol is a suggested method for biotinylating sulfhydryl-containing proteins using iodoacetyl-LC-biotin. The required sulfhydryl groups may be provided through reductive cleavage of disulfide bonds or by the use of thiolation reagents (Chapter 1, Section 4.1). Other molecules may be modified with iodoacetyl-LC-biotin using similar techniques.

Protocol

1. Dissolve the sulfhydryl-containing protein to be biotinylated in 50 m*M* Tris, 0.15 *M* NaCl, 10 m*M* EDTA, pH 8.3, at a concentration of 4 mg/ml.
2. Dissolve iodoacetyl-LC-biotin (Pierce) in DMF at a concentration of 4 m*M* (2 mg/ml). Protect from light.
3. Add 50 µl of the iodoacetyl-LC-biotin solution to each milliliter of the protein solution. Mix well. This level of addition represents a 3.28-fold molar excess of biotinylation reagent over the quantity of protein present if the protein has a molecular weight of 67,000 and possesses one sulfhydryl. Adjustments to the amount of reagent addition may have to be made to be appropriate for other

proteins of different molecular weight. Consideration of the number of sulfhydryls present per protein molecule also should be done. React the biotinylation reagent at no more than a three- to five-fold molar excess over the amount of sulfhydryls present to ensure specificity of the iodoacetyl group for only —SH groups. Higher ratios of reagent to protein may cause reaction with amine groups present on the protein.

4. React for 90 min in the dark at room temperature.
5. Remove excess reactants and reaction by-products by dialysis or gel filtration using a column of Sephadex G-25.

3.3. Carbonyl- or Carboxyl-Reactive Biotinylation Agents

Hydrazide- or amine-containing biotinylation compounds can be used to modify carbonyl or carboxyl groups on other molecules. Hydrazides spontaneously react with aldehydes and ketones to give hydrazone linkages. The hydrazones may be further stabilized by reduction with sodium cyanoborohydride. The amine-containing biotinylation reagents (or the hydrazide ones) may be coupled to carboxylate groups using a carbodiimide reaction (Chapter 3, Section 1). In addition, amine- or hydrazide-containing biotinylation reagents may be coupled to cytosine residues in DNA or RNA by transamination catalyzed by bisulfite (Chapter 17, Section 2.3).

Biotin-Hydrazide and Biotin-LC-Hydrazide

Biotin-hydrazide is *cis*-tetrahydro-2-oxothieno[3,4-*d*]-imidazoline-4-valeric acid hydrazide, the hydrazine derivative of *D*-biotin off its valeric acid carboxylate (Pierce). The hydrazide functional group reacts with aldehyde and ketone groups to give hydrazone linkages. Although formyl groups are not common in biological molecules, they may be created by oxidation of diols with sodium periodate (Chapter 1, Section 4.4). Thus, glycoconjugates may be targeted specifically at their sugar residues. Biotinylation of these oxidized carbohydrates with biotin-hydrazide produces modifications that may be away from active centers or binding sites (Fig. 255). Particularly, immunoglobulins may be biotinylated with this reagent at their polysaccharide groups, which are present in the Fc region of the IgG molecule. Directed modification in this manner avoids the antigen binding sites at the ends of the heavy and light chains, thus preserving antibody activity and allowing avidin or streptavidin probes to dock without blocking or interfering with antigen binding.

Biotin-hydrazide also may be used to couple with carboxylate-containing molecules. Hydrazides can be coupled with carboxylic acid groups by using the carbodiimide reaction (Chapter 3, Section 1). The carbodiimide activates a carboxylate to an *o*-acylisourea intermediate. Biotin-hydrazide can react with this intermediate via nucleophilic addition to form a stable covalent bond.

Biotin-hydrazide has been used to biotinylate antibodies at their oxidized carbohydrate residues (O'Shannessy *et al.*, 1984, 1987; Hoffman and O'Shannessy, 1988), to modify the low-density lipoprotein (LDL) receptor (Wade *et al.*, 1985), to biotinylate nerve growth factor (NGF) (Rosenberg *et al.*, 1986), and to modify cytosine groups in oligonucleotides to produce probes suitable for hybridization assays (Reisfeld *et al.*, 1987) (Chapter 17, Section 2.3).

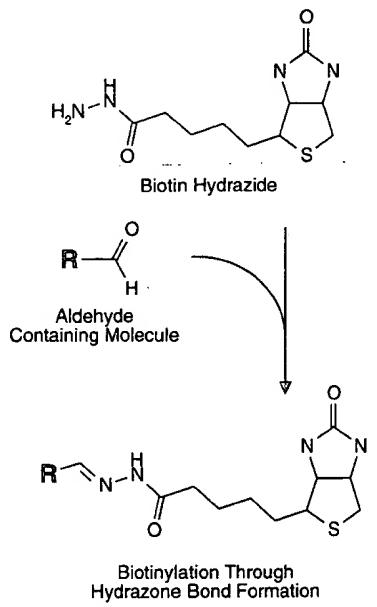
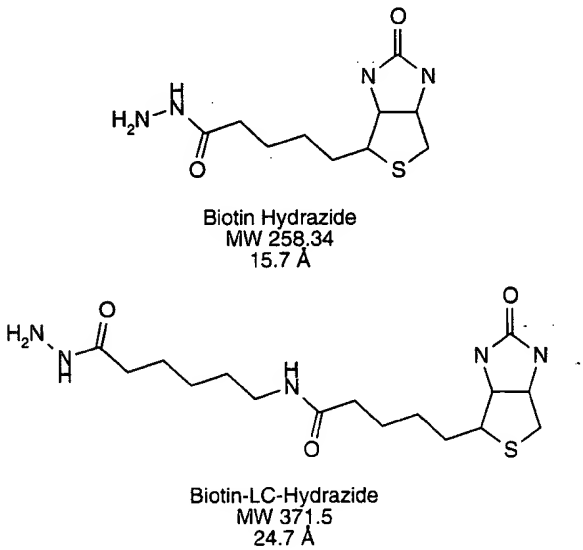


Figure 255 Biotin-hydrazide can be used to label aldehyde-containing molecules, creating hydrazone bonds.



An analog of this biotinylation reagent with a longer spacer arm also exists. Biotin-LC-hydrazide contains a 6-aminocaproic acid extension off its valeric acid group (Pierce). The increased length of this spacer (24.7 Å) provides more efficient interaction potential with avidin or streptavidin probes, possibly increasing the sensitivity of assay systems. The reactions of biotin-LC-hydrazide are identical to those of biotin-hydrazide.

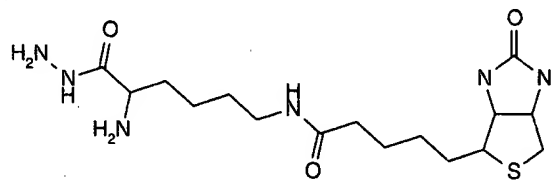
The following protocol describes the use of biotin-hydrazide to label glycosylated proteins at their carbohydrate residues. Control of the periodate oxidation level can result in specific labeling of sialic acid groups or general sugar residues (Chapter 1, Section 4.4).

Protocol

1. Dissolve a periodate-oxidized glycoprotein (i.e, an antibody—see Chapter 10, Section 1.3) in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, at a concentration of 2 mg/ml. Note: The buffer, 0.1 *M* sodium acetate, pH 5.5, is typical of literature references for reacting a hydrazide compound with an aldehyde-containing molecule to form a hydrazone linkage. Alternative buffer conditions using higher pH values also work well. Physiological pH conditions with the use of a reducing agent such as sodium cyanoborohydride (step 4) produce the most efficient labeling conditions when using hydrazide-containing reagents.
2. Add biotin-hydrazide or biotin-LC-hydrazide to a final concentration of 5 m*M*.
3. React for 2 h at room temperature.
4. To reduce the hydrazone bonds to more stable linkages, cool the solution to 0°C and add an equal volume of 30 m*M* sodium cyanoborohydride in PBS. Incubate for 40 min. Note: if the presence of a reducing agent is detrimental to protein activity, eliminate this step. In most cases, the hydrazone linkage is stable enough for avidin–biotin labeling experiments.
5. Remove excess reactants by dialysis or gel filtration using a column of Sephadex G-25.

Biocytin Hydrazide

Another biotinylation reagent that can spontaneously couple with aldehyde- or ketone-containing molecules is biocytin hydrazide (Pierce). Produced by forming the hydrazine derivative of biocytin—a lysine–biotin complex often found naturally in serum (Section 3.1)—the compound has better solubility in aqueous solutions than either biotin-hydrazide or biotin-LC-hydrazide, discussed previously. The solubility enhancement of biocytin-hydrazide is due to the presence of lysine's α-amino group, which is protonated and positively charged at physiological pH. The reagent can be used to label carbohydrate-containing molecules, such as glycoproteins, after they have been oxidized to contain reactive aldehydes (Chapter 1, Section 4.4). The hydrazide group forms a hydrazone linkage with the aldehydes, thus directing the biotinylation reaction toward the polysaccharide regions of glycoconjugates (Fig. 256).



Biocytin Hydrazide
MW 386.51

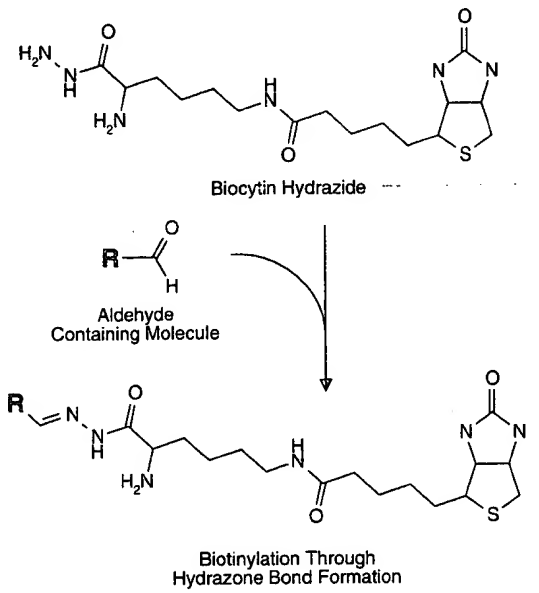


Figure 256 Biocytin-hydrazide reacts with aldehyde-containing molecules to form hydrazone bonds.

Biocytin hydrazide was used to label specifically sialic acid residues and galactose residues, and for general sugar modification (Bayer *et al.*, 1988). The galactose residues were oxidized using galactose oxidase after treatment with neuraminidase (Chapter 1, Section 4.4). The use of this approach for labeling glycoproteins *in situ* was found to be optimal, due to the other potential side reactions that may occur when using sodium periodate.

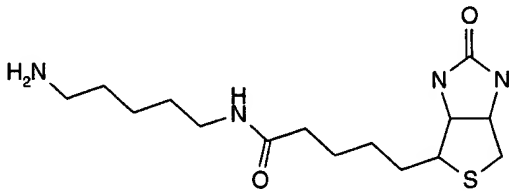
The reactivity and use of biocytin-hydrazide is similar to that described for biotin-hydrazide in Section 3.3. The following protocol for labeling glycoproteins at oxidized carbohydrate (galactose) sites is from Bayer and Wilchek (1992).

Protocol

1. Dissolve the glycoprotein to be labeled in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, containing 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ (labeling buffer), at a concentration of 1 mg/ml.
2. Dissolve biocytin-hydrazide (Pierce) in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4 (PBS), at a concentration of 20 mg/ml.
3. To each milliliter of glycoprotein solution, add 30 µl of neuraminidase (1 unit/ml as supplied by Behringwerke AF), then 30 µl of galactose oxidase (previously dissolved at 100 units/ml in the labeling buffer of step 1), and finally 100 µl of the biocytin hydrazide solution.
4. React for 2 h at 37°C.
5. Remove unreacted reagents by dialysis or gel filtration.

5-(Biotinamido)pentylamine

The derivative 5-(biotinamido)pentylamine contains a 5-carbon cadaverine spacer group attached to the valeric acid side chain of biotin (Pierce). The compound can be used in a carbodiimide reaction process to label carboxylate groups in proteins and other molecules, forming amide bond linkages (Chapter 3, Section 1). However, the main use of this biotinylation reagent is in the determination of factor XIIIa or transglutaminase enzymes in plasma, cell, or tissue extracts.



5-(Biotinamido)pentylamine
MW 328.48

Factor XIII, also known as plasma transglutaminase, is an enzyme of the blood coagulation cascade. It is activated by thrombin and calcium to factor XIIIa, at which point it catalyzes covalent cross-links between the ε-amine group of lysine side chains and the γ-glutamyl side chain of glutamine residues. Abnormal levels of factor XIII in plasma are clinically important, being associated with cancer, liver or renal disfunction, or various bleeding disorders. The assay of transglutaminase activity therefore is important for investigating the activity and function of this enzyme as it relates to post-translational protein modification as well as various disease states.

5-(Biotinamido)pentylamine is able to participate in the acyltransferase reaction, becoming covalently attached to protein substrates at their glutamine residues (Fig. 257). Lee *et al.*, (1988) used this biotinylation reagent to quantify factor XIII in plasma. Transglutaminase activity resulted in the modification of an *N,N'*-dimethylcasein substrate, which was subsequently detected by an avidin–biotin assay procedure. The assay may be done in microplates using wells coated with the substrate protein and quantifying the enzyme activity with streptavidin–alkaline phosphatase (Slaughter *et al.*, 1992). Jeon *et al.*, (1989) subsequently applied the assay to the measurement of transglutaminase activity in cells. Components biotinylated in cellular systems also can be isolated by use of affinity chromatography on immobilized avidin (Lee *et al.*, 1992).

3.4. Photoreactive: Photobiotin

Biotin derivatives containing a photoreactive group provide nonselective biotinylation potential at certain reactive hydrogen sites or nucleophilic groups. They can be used to incorporate an avidin-binding, biotin group into molecules that do not possess amines, sulfhydryls, or other easily modifiable functional groups. Most of these photoreactive derivatives utilize the phenyl azide type of photosensitive group, which can be activated by exposure to UV light to an intermediate nitrene or the nucleophile-reactive dehydroazepine (Chapter 2, Section 7.1, and Chapter 5, Section 3).

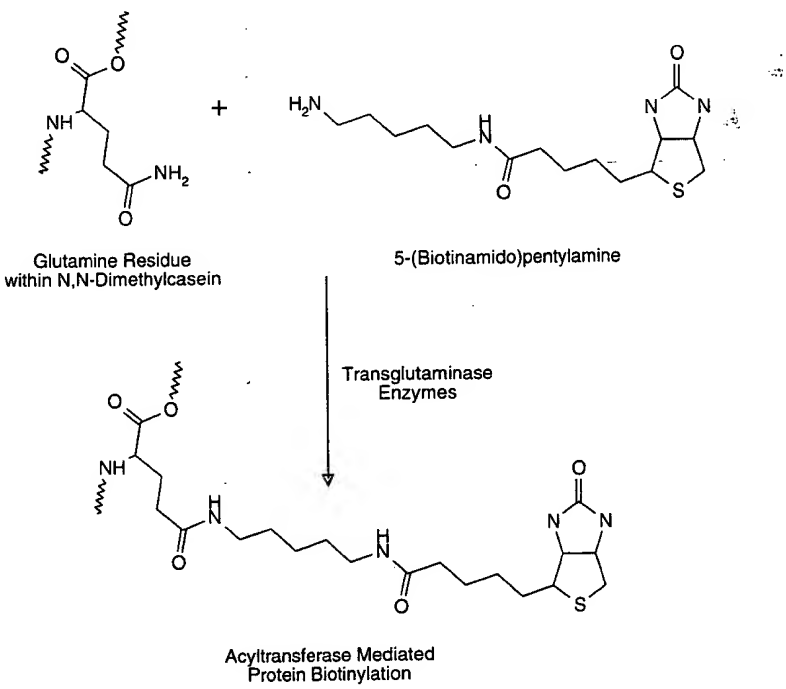


Figure 257 5-(Biotinamido)pentylamine can be used to label glutamine residues in proteins by enzymatic action of transglutaminase.

Perhaps the most common photoreactive biotin derivative is *N*-(4-azido-2-nitrophenyl)-aminopropyl-*N'*-(*N-d*-biotinyl-3-aminopropyl)-*N'*-methyl-1,3-propanediamine, simply called photoactivatable biotin or photobiotin (Forster *et al.*, 1985) (Pierce). The compound contains a 9-atom diamine spacer group on the biotin valeric acid side chain at one end, while the other end of the spacer terminates in the aryl azide functional group. The presence of a nitro group on the phenyl azide ring allows for photoactivation at higher wavelengths approaching the visible region of the spectrum, thus avoiding potential breakdown of biological molecules through UV exposure. Photolyzing with light at a wavelength of 350 nm causes rapid activation with nitrene formation. The nitrene can couple to replaceable hydrogen sites or add to double bonds within Van der Waals distance or undergo ring expansion to the dehydroazepine. If ring expansion occurs, the principal target group for coupling is a nucleophile, such as a primary amine (Fig. 258).

Photobiotin has been used to biotinylate numerous macromolecules, including proteins and nucleic acids. The biotinylation of alkaline phosphatase was done with complete retention of activity (Forster *et al.*, 1985). Tubulin was labeled with photobiotin and detected on dot blots down to a level of 10 pg of sample using an avidin–enzyme conjugate (Lacey and Grant, 1987). DNA and RNA were labeled for use in hybridization assays (Forster *et al.*, 1985; Keller *et al.*, 1989). For instance, photobiotin-modified probes have been used to detect flavivirum RNA in infected cells (Khan and Wright, 1987), to detect single-copy genes and low-abundance mRNA

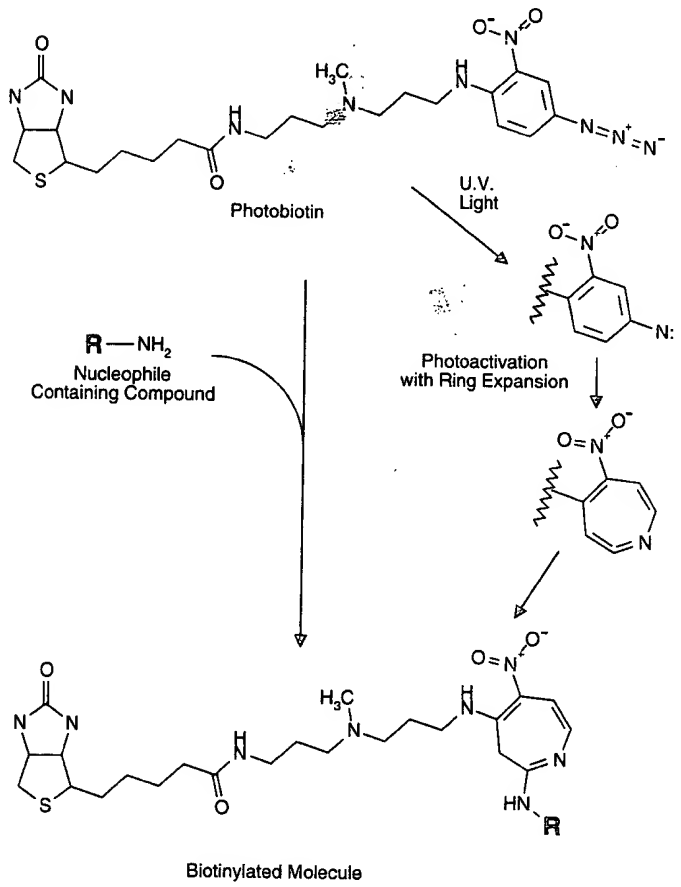
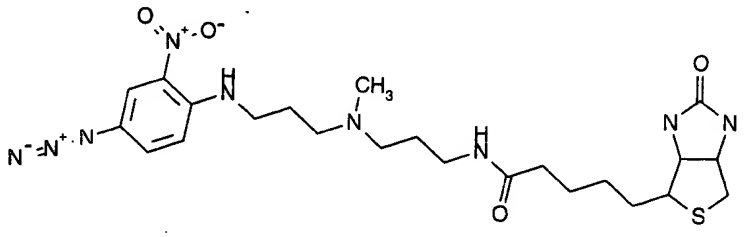


Figure 258 Photobiotin can be made to couple spontaneously with nucleophiles by exposure to UV light. The phenyl azide ring undergoes ring expansion to a highly reactive dehydroazepine intermediate, which can react with amines.

(McInnes *et al.*, 1987), for the diagnosis of barley yellow dwarf virus (Habili *et al.*, 1987), to assay luteinizing hormone β mRNA in individual gonadotropes (Childs *et al.*, 1987), and to perform DNA mapping using a cross-hybridization technique (Chetrit *et al.*, 1989).



Photobiotin
N-(4-azido-2-nitrophenyl)-aminopropyl-
N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine
MW 533.65
30.0 Å

Photobiotin can be dissolved in water or buffer at a concentration of 1 mg/ml and stored in the dark at −20°C until needed. As long as no exposure to light is permitted, the compound is stable for at least 1 year under these conditions.

The protocol for modifying DNA probes with photobiotin can be found in Chapter 17, Section 2.3. It is based on the method of Forster *et al.* (1985). The following method is a suggested protocol for the modification of proteins using a photoreactive biotin derivative. Some optimization may be necessary to obtain the best incorporation levels.

Protocol for Labeling Proteins with Photobiotin

1. Dissolve the protein to be biotinylated at a concentration of at least 1 mg/ml in water or dilute buffer at neutral pH.
2. In subdued light, dissolve photobiotin (Pierce) in water at a concentration of 1 mg/ml.
3. Add a quantity of photobiotin solution to the protein solution to give at least a five-fold molar excess of biotinylation reagent.
4. Place in an ice bath and irradiate from above (about 10 cm away) for 15 min using a sunlamp (such as Philips Ultrapnil MLU 300 W, General Electric Sunlamp RSM 275 W, or National Self-Ballasted BHRF 240–250 V 250 W W-P lamp).
5. Remove excess photobiotin by dialysis or gel filtration using a column of Sephadex G-25 (Pharmacia).

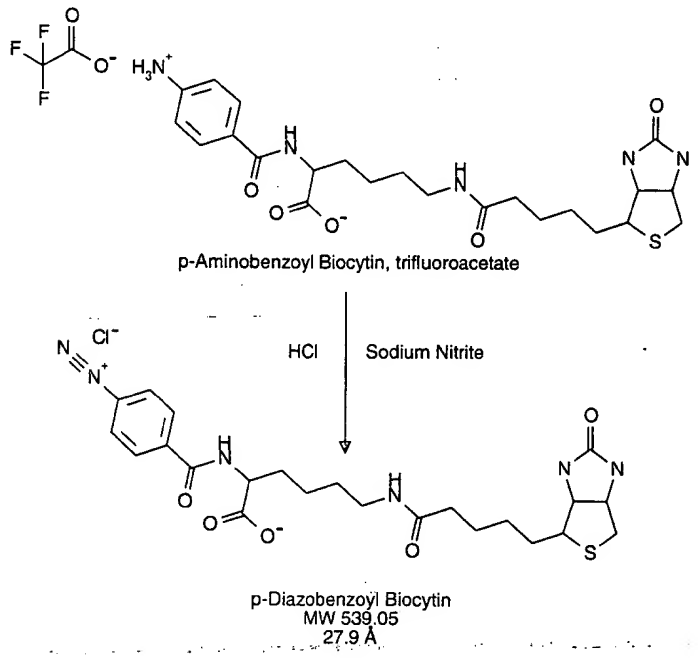


Figure 259 The aminophenyl group of this biotin derivative can be transformed into a diazonium functional group by treatment with sodium nitrite in dilute HCl.

3.5. Active-Hydrogen-Reactive: *p*-Aminobenzoyl Biocytin, Diazotized

p-Aminobenzoyl biocytin contains a 4-aminobenzoic acid amide derivative off the α-amino group of biocytin's (Section 3.1) lysine residue (Pierce). The aromatic amine can be treated with sodium nitrite in dilute HCl to form a highly reactive diazonium group (Fig. 259), which is able to couple with active hydrogen-containing compounds. A diazonium reacts rapidly with histidine or tyrosine residues within proteins, forming covalent diazo bonds (Wilchek *et al.*, 1986) (Fig. 260). It also can react with guanidine residues within DNA at position 8 of the base (Rothenberg and Wilchek, 1988) (Fig. 261). Biotinylation via diazo linkages is reversible by treatment with a 10-fold molar excess of $Na_2S_2O_4$ (sodium dithionite) in 50 m*M* Tris, pH 8.5 (Gorecki *et al.*, 1971) (Chapter 2, Section 6.1, and Chapter 4, Section 9).

The procedure for creating the diazonium derivative of *p*-aminobenzoyl biocytin and coupling to a protein or a nucleic acid is as follows.

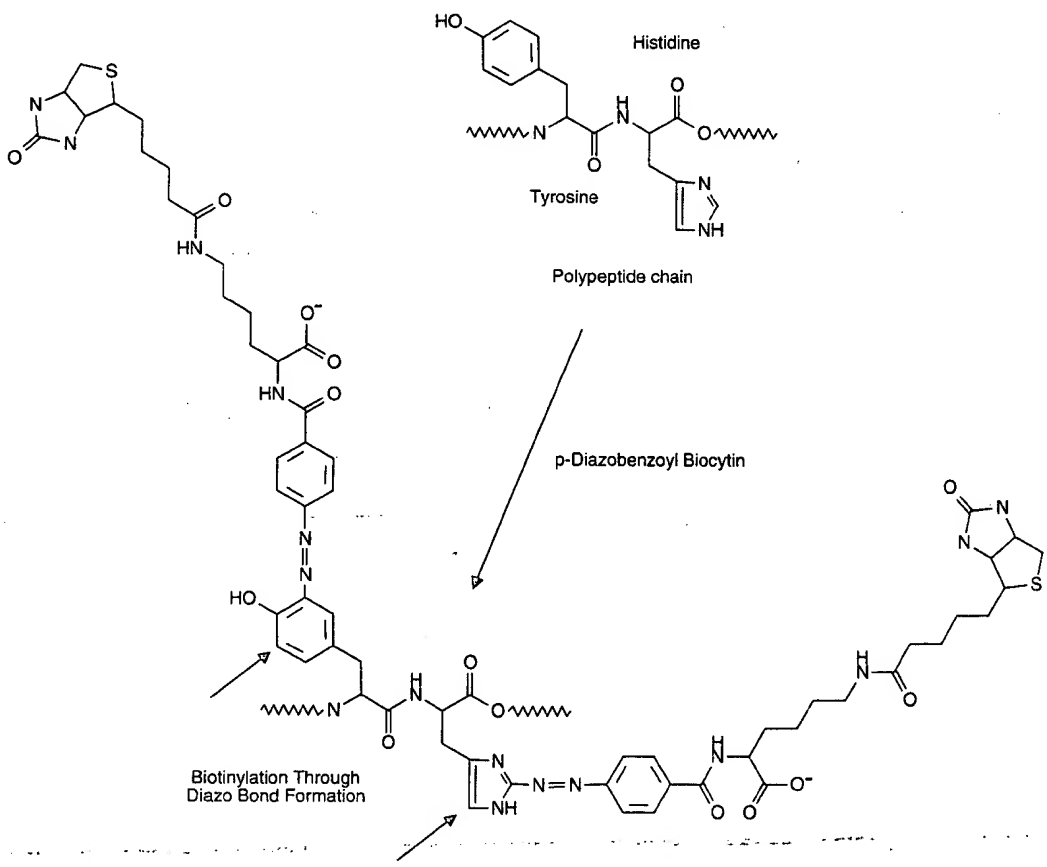
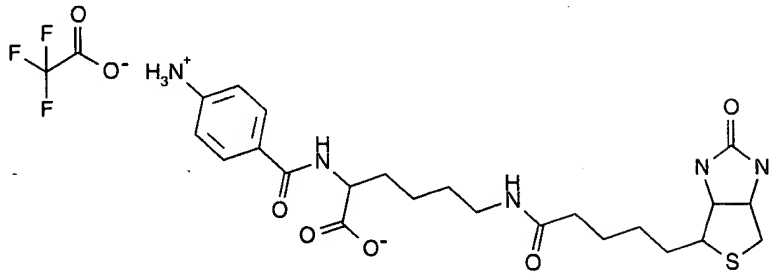


Figure 260 The diazonium group of *p*-diazobenzoylbiocytin can react with tyrosine or histidine residues in proteins to form diazo bonds.

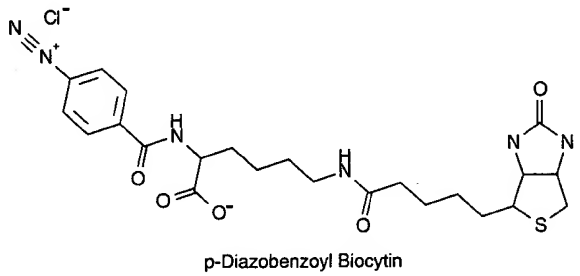


p-Aminobenzoyl Biocytin,
trifluoroacetate salt
MW 605.63

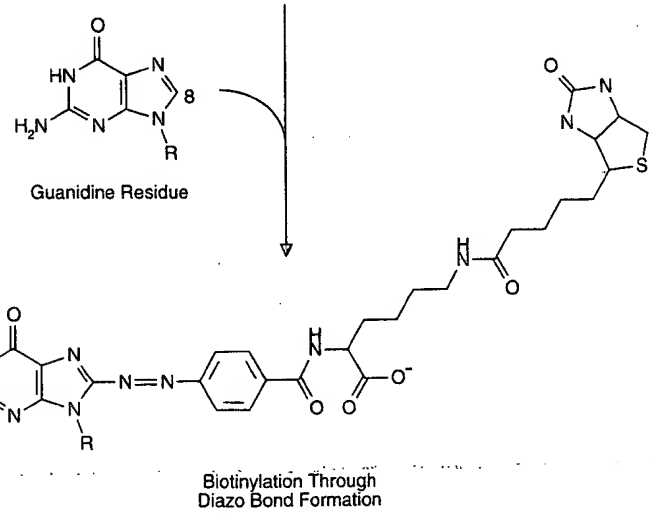
Protocol

Formation of the Diazonium Derivative

1. Dissolve 2 mg of *p*-aminobenzoyl biocytin (Pierce) in 40 µl of 1 *N* HCl (concentration of 50 mg/ml). Cool the solution in ice.



p-Diazobenzoyl Biocytin



Guanidine Residue

Biotinylation Through
Diazo Bond Formation

Figure 261 The diazonium group of *p*-diazobenzoylbiocytin can couple to the C-8 position of guanidine bases in nucleic acids, forming diazo bonds.

2. Dissolve 7.7 mg of sodium nitrite in 1 ml of ice-cold water. Prepare fresh.
3. Mix 40 µl of the *p*-aminobenzoyl biocytin solution with 40 µl of the sodium nitrite solution.
4. React for 5 min on ice to create the diazonium derivative.
5. Stop the reaction by the addition of 35 µl of 1 *N* NaOH. Use immediately for biotinylation.

Biotinylation of Proteins on Blots Using the Diazonium Derivative of *p*-Aminobenzoyl Biocytin

1. Dilute the diazonium derivative of *p*-aminobenzoyl biocytin prepared in preceding part with 0.2 *M* sodium borate, pH 8.4, to a concentration of 10 µg/ml.
2. Transfer proteins onto a nitrocellulose membrane using any appropriate procedure, including dot blotting the protein solution onto the surface.
3. Incubate the membrane with the biotin derivative at a ratio of 1 ml/cc^2 of membrane.
4. React for 1 h at room temperature.
5. Wash the membrane thoroughly with 0.1 *M* Tris, 0.15 *M* NaCl, pH 7.5.
6. Block nonspecific sites on the membrane with an appropriate blocking component (such as BSA) and detect the biotinylated proteins using an avidin or streptavidin conjugate.

4. Iodination Reagents

Modification of proteins and other molecules with a radioactive element provides a means of detection that can be extremely sensitive for assay, localization, and imaging applications. Among the most common radiolabels for biological studies are ^{14}C, ^{32}P, ^{35}S, and ^{3}H, and the isotopes of iodine, ^{125}I and ^{131}I. The unstable isotopes of carbon, phosphorus, sulfur, and hydrogen are all β emitters, releasing particulate radiation consisting of either positrons or electrons. To measure labeled molecules containing β emitters often necessitates tedious sample manipulation including tissue homogenization and mixing with scintillation cocktails for subsequent counting.

The radioactive isotopes of iodine, by contrast, are both γ emitters, providing a much easier route to measurement than β-particle-emitting radioisotopes. High-energy electromagnetic radiation can be detected directly without the need for intermediate scintillation cocktails. Iodine-131 was the first unstable iodine isotope to be used for labeling protein molecules (Li, 1945; Pressman and Keighley, 1948). The ^{131}I isotope decays by both β (electron) and γ emission. The specific activity of this element can be as high as 6550 Ci/mmol, providing extraordinary sensitivity for detecting labeled molecules.

Iodine-125 decays by electron capture followed by γ emission. However, the maximum energy of ^{125}I electromagnetic energy emission can be as little as one-tenth to one-third that of ^{131}I (Wilbur, 1992; Powsner, 1994). The greater energy intensity of ^{131}I emission actually can be a disadvantage, since γ rays emanating from it are more penetrating, requiring increased precautions and greater protective equipment. In addition, the relatively short half-life of ^{131}I (8.1 days) compared to that of ^{125}I (6

days) necessitates that labeled compounds be prepared more often, since activity losses will be severe on storage. Because ^{125}I is not a particulate emitter, its use *in vivo* for imaging applications limits radiation damage to surrounding proteins, cells, and tissues.

These factors make ^{125}I the iodine label of choice for radiolabeling biological molecules. Its commercial availability from a number of suppliers at relatively low cost further adds to its popularity. Even though it has lower specific activity than ^{131}I, iodine-125 still provides much greater sensitivity than ^{14}C, ^{32}P, ^{35}S, or ^{3}H in labeling biomolecules. In fact, the use of a radioactive iodine label can create probes that have 150-fold more sensitivity than tritiated molecules and as much as 35,000 times the detectability of ^{14}C-labeled molecules (Bolton and Hunter, 1986).

Radioiodination is the process of chemically modifying a molecule to contain one or more atoms of radioactive iodine. Early studies on protein modification determined that iodine in aqueous solution formed a reactive ion, H_2OI^+ (Fig. 262), that is capable of modifying tyrosine side chains and the imidazole groups of histidine, and either modifying sulfhydryl groups or catalyzing their oxidation to disulfides (Fig. 263). More modern methods utilize a chemical agent to create the reactive iodine species, thus driving the reaction at much greater rates.

There are two main methods of radioiodination commonly employed to modify proteins and other molecules: (1) direct labeling of the desired protein or other target molecule in the presence of an oxidizing agent or (2) indirect labeling of the desired molecule by first labeling an intermediate compound, which is then used to perform the final modification. Direct labeling methods are by far the most common, and the chemical reactions used in this process have been reviewed (Regoeczi, 1984).

The prevailing procedures for direct coupling of ^{125}I to a protein or other molecule is through the use of an oxidizing agent. The *in situ* preparation of an electrophilic radioiodine species is fundamental to the ability to modify certain reactive sites within the desired molecules. The most common oxidizing compounds are *N*-haloamine derivatives, such as *N*-chlorotoluenesulfonamide (chloramine-T) or 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN). In most instances, such compounds do not harm the proteins being labeled, although careful control over reaction times should be done to prevent overlabeling or oxidative damage. A secondary method of producing an oxidative effect is to use an enzyme-driven system. The glucose oxidase/lactoperoxidase reaction creates reactive iodine through the production of hydrogen peroxide from glucose with the subsequent action of peroxidase to form I_2 from I^-.

Formation of the electrophilic halogen species leads to the potential for rapid reaction with compounds containing strongly activating groups, such as activated aryl compounds. Particularly, substances containing aromatic ring structures that have

$$I_3^- \rightleftharpoons I_2 + I^-$$

$$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$$

Figure 262 Iodide anion in aqueous solution undergoes an equilibrium reaction process to form the reactive H_2OI^+ species.